AWARD NUMBER: W81XWH-13-1-0372

TITLE: Development of Novel p16INK4a Mimetics as Anticancer Therapy

PRINCIPAL INVESTIGATOR: Mark Klein, M.D.

CONTRACTING ORGANIZATION: Minnesota Veterans Medical Research and Education Foundation
Minneapolis, MN 55417

REPORT DATE: October 2014

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual Report	15 Sep 2013 - 14 Sep 2014

4. TITLE AND SUBTITLE

Development of Novel p16INK4a Mimetics as Anticancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0372

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Mark Klein, M.D.

E-Mail:mark.klein2@va.gov

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Minnesota Veterans Medical Research and Education Foundation
1 Veterans' Dr., Research 151
Minneapolis, MN 55417-2309

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Mesothelioma therapy is a highly fatal disease that has poorly effective therapy with dose-limiting side-effects. Low expression of the CDK4/CDK6 inhibitor p16INK4a has been demonstrated in up to 90% of mesothelioma tumors. The objective of this application as a next step in the pursuit of this long term goal is to identify stabilized peptides that will mimic the interaction between p16INK4a and CDK4/6. The central hypothesis of this proposal is that protein-protein interactions can be replicated or disrupted by stabilized peptides that have been identified via the identification of pharmacophores of small peptides that interact with CDK4/6. The specific aims are as follows. (1) Determine structure-function relationships of overlapping peptides derived from p16INK4a that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. (2) In vitro functional studies will be used to evaluate bioactivities of stabilized peptides. (3) In vitro ADME studies studies to evaluate the cell permeability, delivery, and efficacy of stabilized peptides.

15. SUBJECT TERMS
Mesothelioma, CDK4, CDK6, peptides, protein-protein interactions

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	19	19b. TELEPHONE NUMBER *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

Mesothelioma therapy is a highly fatal disease that has poorly effective therapy with dose-limiting side-effects. Low expression of the CDK4/CDK6 inhibitor $p16^{INK4a}$ has been demonstrated in up to 90% of mesothelioma tumors. Replacement of $p16^{INK4a}$ activity in laboratory models has demonstrated activity against CDK4 and CDK6, tumor response, and increase in survival in xenograft models. The long term goal of this laboratory is to identify key principles of drug discovery that will allow the appropriate and selective disruption or replacement of protein-protein interactions via stabilized peptides. The objective of this application as a next step in the pursuit of this long term goal is to identify stabilized peptides that will mimic the interaction between $p16^{INK4a}$ and CDK4/6. The central hypothesis of this proposal is that protein-protein interactions can be replicated or disrupted by stabilized peptides that have been identified via the identification of pharmacophores of small peptides that interact with CDK4/6. The specific aims are as follows. (1) Determine structure-function relationships of overlapping peptides derived from $p16^{INK4a}$ that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. (2) *In vitro* functional studies will be used to evaluate bioactivities of stabilized peptides. (3) In vitro ADME studies to evaluate the cell permeability, delivery, and efficacy of stabilized peptides. We are using structural biology, computational modeling, *in vitro* efficacy, and *in vitro* ADME studies to design and evaluate anti-mesothelioma peptides. Work funded by this grant opportunity will lead to progress in targeted therapy with the potential for fewer side effects and higher efficacy.

Key words

peptide
mesothelioma
cell cycle
cyclin-dependent kinase (CDK)
protein-protein interaction
molecular dynamics
nuclear magnetic resonance (NMR)

ACCOMPLISHMENTS

Major Goals of the Project (as defined in the Statement of Work)

Statement of Work

Specific Aim #1. Determine structure-function relationships of overlapping peptides derived from $p16^{INK4a}$ that inhibit the activity of CDK4/6 and identify stabilized peptides that inhibit CDK4/6. The pharmacophore(s) of mutated peptides from $p16^{INK4a}$ will be assessed via time-resolved fluorescent resonance energy transfer assays and nuclear magnetic resonance spectroscopy to elucidate which residues are important to bind CDK4 and/or CDK6. Stabilized peptides will be evaluated for inhibitory activity toward CDK4/6.

1. Task 1. Identification of the amino acid substitutions that enhance peptide. (Months 1-15)

a. Molecular dynamics analysis of proposed peptides. (Months 1-4) 50% accomplished
b. Analysis of stapled derivatives from peptides p16_10 and p16_2020. (Months 3-6) 50% accomplished
c. Determination of the 3-D structure of the most active peptides via NMR. (Months 6-12) 40% accomplished
d. Enzyme kinetic analysis of the active peptides discovered. (Months 13-15) 0% accomplished

Milestone #1. Identification of all pharmacophores and peptides by the end of year 1.5. 50% accomplished

Specific Aim #2. *In vitro* functional studies will be used to evaluate bioactivities of peptidomimetics and stabilized peptides.

2. Task 2. Determination of the intracellular effects of peptides and compounds determined in Aim 1. (Months 13-24)
    a. Determination the ability of $p16^{INK4a}$-mimetic peptides to enter cells (Months 13-15) 0% accomplished
    b. Determination of the cell growth inhibitory activity of mimetics of $p16^{INK4a}$. (Months 16-18) 25% accomplished
    c. Evaluate how $p16^{INK4a}$ mimetics inhibit Rb phosphorylation in vitro. (Months 19-21) 25% accomplished
    d. Evaluation of efficacy of p16ink4a mimetics to prevent cell cycle progression into S-phase. (Months 22-24) 0% accomplished
    e. Determine if apoptosis is a mechanism of cell death after treatment with peptidomimetics. (Months 22-24). 10% accomplished

Milestone #2. Identification of the intracellular activity of small molecules and peptides by the end of year 2. 20% accomplished

Specific Aim #3. *In vitro* ADME studies to evaluate the cell permeability and potential delivery of peptidomimetics and stabilized peptides.

3. Task 3. ADME studies of compounds and peptides evaluated in aims 1 and 2. (Months 13-24) 0% accomplished
    a. Determine the metabolic stability of identified peptides and identification of responsible enzymes in metabolism of the peptides. (Months 16-18) 0% accomplished
    b. Cell permeability and transport studies of stapled peptides (Months 19-21) 0% accomplished
    c. Determination of plasma protein binding of identified peptides. (Months 22-24) 0% accomplished

What was accomplished under these goals?

Aims 1 and Aims 2 were pursued in parallel. The % accomplishment is listed after each goal. For simplicity, we started analysis of peptides below in cellular proliferation assays due to logistics of availability of materials in the lab when starting experiments. In addition, in order to

inform our decisions and analyses on design of further peptides, we wanted a sense of how well the peptides may get into cells. We focused on the following peptides in addition to a control compound, PD0332991, which is a specific competitive inhibitor of CDK4 and CDK6.

Table 1. Relevant peptide sequences.	
p16_10	FLATLVVLHR
p16_20	DAAREGFLDTLVVLHRAGAR
TAT-p16 20mer	YGRKKRRQRRRDAAREGFLDTLVVHRAGAR
TAT-p16 10mer	YGRKKRRQRRRGFLDTLVVHR
stapled p16_10	FLA(*)LVV(*)HR
stapled p16_20	DAAREGFLA(*)LVV(*)HRAGAR

Below is a summary table of IC50 values encompassing multiple experiments (including from before and after the grant started – including all for simplicity sake and flow of the information). The first 2 columns reflect the IC_{50} values derived from TR-FRET-based assays. These assays are used to evaluate the inhibition of CDK4 and CDK6 by the respective peptides or compound. Curves were fit to 3 separate multiparameter sigmoid curve equations using the program Origin. Below represents only one value of at least 3 derived from the curve fits. Of note, the assay has been quite robust in the past. When getting new supplies for this project, we did not at first see reliable curves, so a significant amount of time was spent to re-standardize the assay to increase the reliability of the assay. The values for PD0332991 had the most variability between equation fits, while the peptides were very similar across the board, aside from some variability in the CDK6 assay for the Tat 20mer. These will be re-analyzed.

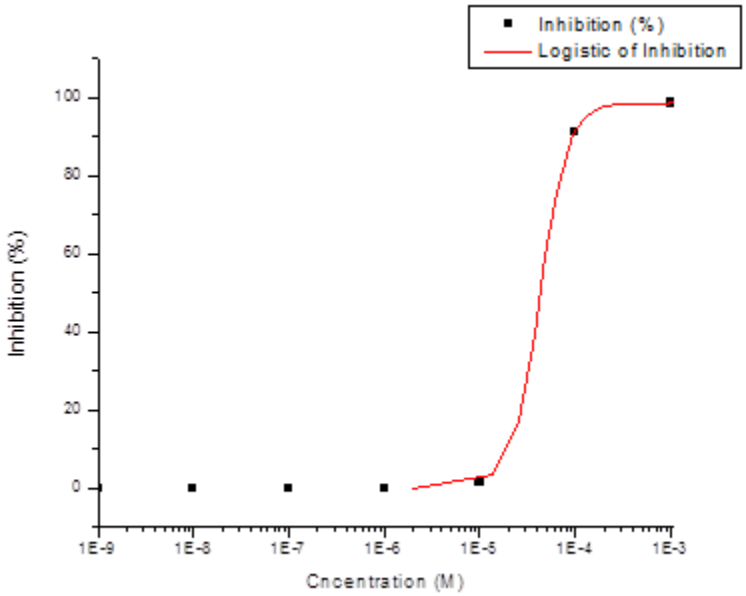
IC_{50}

Peptide	**CDK4**	**CDK6**	**2373**	**2461**	**2596**
10mer	21.2 μM	20.8 μM	> 1 mM	> 1 mM	> 1 mM
20mer	19 μM	14.6 μM	> 1 mM	> 1 mM	> 1 mM
Tat 10mer	2.47 μM	not done	33.3 μM	37.6 μM	44.2 μM
Tat 20mer	860 nM	257 μM	42.7 μM	88.2 μM	43.2 μM
Stapled 10mer	55.3 μM	105 μM	> 1mM	> 1mM	> 1mM
Stapled 20mer	49 μM	87.4 μM	> 1mM	> 1mM	> 1mM
PD0332991	9.48 nM	97.5 nM	1.6 μM	Being analyzed	26.9 μM

The 3 final columns represent the IC_{50} values for the peptides or compound as tested in 3 separate mesothelioma cell lines. The 10mer, 20mer, Tat 10mer, and Tat 20mer reflect peptides that are not modified. The Tat refers to a leader sequence that is recognized to increase cell permeability of peptides. These sequences dramatically increased the efficacy of the peptides, as expected from previous studies done by Dr. Robert Kratkze. In contrast to our previous experience, the stapled peptides did not enter into the cells well. These experiments were done with a new batch

of peptides obtained from New England Peptide, Inc. There was significant precipitation at high concentrations (above 100 μM) that was not seen with previous batches of stapled peptide. An acetyl group was added to the new batch of peptides by the company. This is done frequently by many peptide companies to increase cell permeability. We suspect that in this case, it may promote peptide aggregation. NMR experiments utilizing diffusion gradients to determine aggregation status are underway, but take time due to the technical nature and fact that they are only done by certain groups. We are receiving assistance from the Dr. Kevin Mayo group in this endeavor. New experiments will be done with non-acetylated peptides.

Below is a representative inhibition curve of the Tat 20mer peptide in mesothelioma cell line 2373.



We have NMR data that was collected prior to the start of the grant that show the native 10mer is a helix (previously published. We also have data obtained prior to the grant that the native 20mer has characteristics of a helix (chemical shift data). Data analysis of the final structure is underway. In addition, we have also started NMR experiments of the stapled peptide 10mer during this grant. We have conducted a TOCSY (total correlation spectroscopy) 2-dimensional experiment that shows good resolution of the peptide in solution in the 1D aspect. Data analysis is underway and a NOESY will be done to aid in the structure determination.

In addition, we have molecular dynamics studies underway. Dr. Yuk Sham has assisted with these utilizing the Minnesota Supercomputing Institute. Even though we can have NMR-derived structural evidence that a peptide forms a helical structure, a peptide can be quite mobile in solution, potentially sampling multiple conformations. Molecular dynamics can simulate this motion, to see if the propensity is to stay in a particular conformation, or multiple conformations. One way to determine this is to analyze dihedral angles of each representative snapshot of a simulation. Below is a table from a sample snapshot from a brief simulation of the native 10mer sequence. It reflects a helical formation in 7 of the 10 residues. Simulations are underway for the other peptides at this time. We have several computer scripts underway and ready to go after a lot of work with Dr. Sham and can proceed quickly now through the peptides in a few weeks.

Snapshot 5				In Helical Range (1=yes, 0=no)	
Residue	**Phi**	**Psi**	**Sum**	**Phi**	**Psi**
F		8.6	8.6		0
L	-101.4	-13	-114.4	0	0
A	-87.8	-19.5	-107.3	1	1
T	-45.8	-48.8	-94.6	1	1
L	-70	-24	-94	1	1
V	-77.9	-25	-102.9	1	1
V	-83	-46.7	-129.7	1	1
L	-71	-37.2	-108.2	1	1
H	-109.3	-61.4	-170.7	0	1
R	-76.2		-76.2	1	
			Total	**7**	**7**

Western Blots performed to probe for phosphorylated Rb and actin (+/- peptide) from mesothelioma cell lines. In total, we have data for the following peptides The 4-7th columns are what antibodies (in the case of S795, T821, and T826, antibodies to which particular phosphorylated Rb. We have listed the peptides by molecular weight. Some of the TAT-peptides definitely had some activity resulting in decreased Rb phosphorylation. The native peptides did not show a decrease in Rb phosphorylation. We are still analyzing some of the stapled peptides in new experiments.

Peptide* (molecular wt.)	Conc. of peptide	Duration of treatment	Antibodies tested^{+}			
1168	100 uM	72 hr.	S795	T821	T826	actin
2124	100 uM	24 hr.	S795	T821	T826	actin
1205	100 uM	72 hr.	S795	T821	T826	actin
2202	100 uM	72 hr.	S795	T821	T826	actin
2767	25 uM	24 hr.	S795	T821	T826	
2767	50 uM	24 hr.	S795	T821	T826	actin
2767	75 uM	24 hr.	S795	T821		actin
3727	25 uM	24 hr.	S795	T821		
3727	100 uM	24 hr.	S795			

* 1168 – native 10mer, 2124 – native 20mer, 1205 – stapled 10mer, 2202 – stapled 20mer, 2767 –pTAT 10mer, 3727 –pTAT 20mer

$^{+}$letter and number indicate phosphorylated site on Rb protein

Here is an example of a blot done before the grant started in 9/15/14 (from palbociclib tested earlier in 2014):

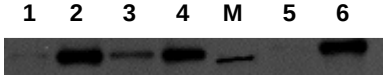


Figure 4 Immunoblotting results. Lanes 1, 3 and 5 are treated with palbociclib, while lanes 2, 4, and 6 showed untreated cells. M, marker.

o

Methods:

Mesothelioma Cell lines. The mesothelioma cell lines used in this study (H 2373, H2461 and H2596) were obtained from either the ATCC (American Type Culture Collection) or in collaboration with Dr. Frederick Kaye (National Cancer Institute)[1]. All mesothelioma cell lines express wild-type pRb and lack functional $p16^{INK4a}$. The cells were grown in RPMI-1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA), 10 mM HEPES (Corning, Manassas VA), 1 mM sodium pyruvate(Sigma, St. Louis, MO, USA) , 1.5% sodium bicarbonate (Sigma, St. Louis, MO, USA), and 1x concentration of antibiotic/antimycotic reagent (Gibco BRL, Grand Island, NY, USA) at 37^o C and 5% CO_2.

$p16^{INK4a}$Peptides and PD 0332991. Several peptides containing amino acid residues from $p16^{INK4a}$, which constitute the Cdk 4/6 inhibitory sequence, were used. These included the native 10 residue sequence (FLATLVVLHR-NH_2) and the native 20 residue sequence (DAAREGFLATLVVLHRAGAR-NH_2). In addition, the native sequences were each linked to a protein transduction domain (TAT) of the HIV-1 virus (YGRKKRRQRRRGFLATLVVLHR-NH_2 and YGRKKRRQRRRGDAAREGFLATLVVLHRAGAR-NH_2). These four peptides were synthesized at the BioMedical Genomics Center at the University of Minnesota (Minneapolis, MN, USA). Two stabilized (stapled) versions of the native peptides ([Cyc(4,8)] H_2N-FLA(S5)LVV (S5)HR-0H and [Cyc(10,14)] Ac-DAAREGFLA(S5)LVV(S5)HRAGAR-OH) were manufactured by New England Peptide (Gardner, MA, USA). All peptides were dissolved in DMSO and then diluted to a standard stock solution of 10mM peptide in 5% DMSO in dH_20 and stored at -70^o C. The Cdk 4/6 inhibitor, PD 0332991 isethionate, was obtained from Sigma, St. Louis, MO, USA.

Antibodies. Various antibodies were used including anti-Rb , anti-Rb (phospho S795), anti-Rb (phospho T821), anti-Rb (phospho T826), horse radish peroxidase-conjugated goat/anti-rabbit (Abcam, Cambridge, MA, USA); anti Rb, horse radish peroxidase-conjugated goat/anti- mouse (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-actin (Sigma, St. Louis, MO, USA).

Cell proliferation assays. Live cells, as determined by Trypan blue dye exclusion assay, were counted on a hemocyometer and plated on 96-well plates (3000 cells/100ul/well) in the RPMI media (including supplements)[1]. After 24 hours, 10 ul of the various $p16^{INK4a}$ peptides in varying concentrations were added to the wells and gently vortexed to mix. After a 72 hr incubation at 37^o C and 5% CO_2, 10 ul of solution from Cell Counting Kit – 8 (Dojindo Laboratories, Kunamoto, Japan) was added to each well and the plates were incubated for 2 hr. The plates were read at 450 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Because addition of the peptides created a precipitate in the higher concentrations (i.e. 1 mM and 100 uM), which interfered with the optical readings, the contents of the wells containing a precipitate were placed in a microfuge tube and spun for 15 seconds in a microcentrifuge at 14,000 rpm. The supernatant was removed and read again in the microplate reader. The reduction in optical density represented the reduction in mitochondrial succinate dehydrogenase activity, hence the reduction in surviving cell numbers.

Kinase assay. The effect of the peptides on the kinase activity of Cdk4 and Cdk6 was evaluated using a commercially available time-resolved fluorescent resonance energy transfer (TR-FRET) assay[2]. The Adapta Universal Kinase Assay (Invitrogen Corporation, Carlsbad, CA 92008) measures kinase activity by correlating ADP formation with substrate phosphorylation. In the first phase, a kinase reaction is created by placing a kinase, the substrate (Rb), ATP, and various concentrations of a peptide into wells of a 396 well plate for 60 minutes. After the reaction, a detection solution consisting of a europium labeled anti-ADP antibody, an Alexa Fluor 647-labeled ADP tracer, and EDTA is added to the well. In the absence of an inhibitor, ADP formed by the kinase reaction will displace the labeled ADP tracer from the antibody, resulting in a decrease in the TR-FRET signal. In the presence of an inhibitor, the amount of ADP formed is reduced and the resulting intact antibody-tracer interaction results in a higher TR-FRET signal. After a 30 minute equilibration period, the signal was read using a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA, USA).

Effect of Peptides on cellular proteins. Live cells, as tested and counted above, were plated on 6 well plates (1.0×10^6 cells/well for 24 hour assays and 0.15×10^6cells/well for 72 hour assays). On day 2, the cells were aspirated and usual medium with the addition of 1% DMSO and various concentrations of the different peptides were placed in the wells. The cells were incubated for either 24 or 72 hours at 37^o C and 5% CO_2, then harvested and processed for Western blot analysis.

Immunoblotting. Cells were removed from the plates by trypsinization, washed in phosphate-buffered saline (PBS) and lysed in cold lysis buffer (50 mM Tris, pH 7.4 and 1% Triton X-100 containing 1x protease inhibitor mixture (Sigma, St. Louis, MO), 1x phosphatase inhibitor mixture (Sigma, St. Louis, MO) and 1 mM PMSF) for 10 minutes[1]. Cellular debris was pelleted by centrifugation in a microcentrifuge at 14,000 rpm at 4^o C for 10 minutes. The supernatant (lysate) was stored at -70^o C. Protein content was determined by Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). For Rb Westerns, twenty to thirty micrograms of protein was mixed with equal volumes of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and placed in boiling water for 2 minutes. Samples were subjected to electrophoresis on 7.5% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. For actin, 3 micrograms of protein was subjected to electrophoresis on 12% gels and transferred to PVDF membranes. Membranes were rinsed in Tris buffered saline with 0.05% Tween 20 (TBS-T) and incubated in blocking buffer (5% bovine serum albumin (BSA)in TBS-T) for 1 hour at 4^o C (Rb blots) or overnight (actin blots). Rb blots were then incubated in primary antibodies diluted in TBS-T containing 0.5% BSA, overnight at 4^o C. Actin blots were incubated in primary antibody for 1 hour at room temperature. The blots were washed 6x with TBS-T and then incubated with an appropriate secondary antibody, diluted in TBS-T for 1 hour at room temperature. Following 6 washes in TBS-T, blots were developed with a chemiluminescence reagent (Pierce ECL Western Blotting Substrate, Thermo Scientific, Rockford, IL, USA or Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Buckinghamshire, UK) and exposed to x-ray films. X-ray film was processed on an Agfa-CP1000 developer.

NMR. Two-dimensional NMR spectra of the most active peptide derived from peptides identified in Aims 1a and 1b are being obtained[3-8]. TOCSY (total correlational spectroscopy) spectra (to assign peaks to specific amino acids)[41] and NOESY (nuclear Overhauser Effect

Spectroscopy) spectra (to determine through-space interactions < 5 Å) will be obtained and used to computationally model the peptide structure[42].The program NMRPipe[43] will be used to process data, and the program Sparky[44] will be used to evaluate spectra and assign peaks. The program X-PLOR will then be used to perform structural calculations and refinements based on NOE internuclear distant constraints, as mentioned above[45]. The result of this subaim will yield molecular models of the three-dimensional structure of the peptides based on the NMR-derived data.

What opportunities for training and professional development has the project provided?

Coursework: In the spring semester of 2014, I took the graduate level course "Biochemical Aspects of Normal and Abnormal Cell Growth and Cell Death" at the University of Minnesota (offered by the Microbiology, Immunology, and Cancer Biology graduate program). I took this course for credit (2 credit hours) and received a grade of A.

Seminars attended:

I have regularly attended the weekly seminar series offered by the Medicinal Chemistry Department at the University of Minnesota.

Poster presented at the Annual Protein Society Meeting, July 2014:

Some of the work accomplished on this grant was presented at the Annual Meeting of the Protein Society, July 22-25, 2014.

How were the results disseminated to communities of interest?

I presented a talk at our local VA Primary Care Service line on this research January 23, 2014 and to the Division of Hematology, Oncology, and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, MN on January 24, 2014.
I presented a poster at the Annual Meeting of the Protein Society, July 22-25, 2014.

IMPACT

What was the impact on the development of the principal disciplines of the project?

We have identified that TAT-p16 peptides have good potency against CDK4. These were nearly as potent against mesothelioma cell lines as PD0332991 (palbociclib). Stapled peptides had less efficacy against CDK4/6 than anticipated. We suspect this may be due to the olefin tether interfering with the binding of the peptide to CDK4/6. In addition, the peptides did not enter into the cells as they previously have. That has lead us to hypothesize that we need to identify more properties with mesothelioma cell biology before designing several new peptides. We are continuing to analyze peptide mutations now.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS

Changes in approach or reasons for change.

We have not changed any of the aims. Due to some difficulty with solubility and also with developing the necessary programs for molecular dynamics studies, we chose to also pursue parts of aim 2 in parallel with the goal of still having the vast majority of aim 1 finished by the 1.5 year mark or so.

Actual or anticipated problems or delays and actions or plans to resolve them.

Initially, we had more difficulty with usual with solubility for stapled peptides. It appears to have aggregated in solution. We have been systematically adjusting solution conditions to see if this affects the peptides. Of note, the company placed acetyl groups on the n-terminus as part of protocol. Our previous peptides did not have these. We are in the midst of obtaining new peptides to determine if this will affect solubility. We are also working with the Kevin Mayo lab at the University of Minnesota in conducting NMR diffusion gradient experiments to determine aggregation properties (this technique can help to establish the presence of dimers, tetramers, etc. in certain situations). However, this is a specialized technique that takes time to establish for each peptide.

We are moving forward with molecular dynamics studies steadily now that we have most of the program issues worked out in Desmond at the University of Minnesota Supercomputing Institute. The molecular dynamics were a bit delayed due to needing to get special access to the Supercomputing Institute and to get the appropriate amount of computer time. It also took some time to develop software plug-in scripts to work with the program Desmond. Now that is going well and we should have several more peptides analyzed in a few weeks.

We have also had some difficulty with immunoblotting recently – this seems to have been due to age of some of the membranes needed and need for new lysate – these are working much better now.

The primary mentor, Robert Kratzke, M.D., also was unexpectedly gone for just short of 3 months in the spring of 2014 due to unusual circumstances. Prior to that time and after, the PI interacted regularly with Dr. Kratzke. He is now back working full time.

Changes that had a significant impact on expenditures.

We have carried over a small amount of funding from year 1 to year 2 due to issues with peptide solubility. It is anticipated that with changes in protocol that these studies will be finished in the first half of year two. This was a very small percent of the budget.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Not applicable and nothing to report.

PRODUCTS

The PI produced one journal article, one poster at a major meeting, and two oral presentations since beginning the grant. They are listed below. The manuscript is in the appendix.

Journal Article

Klein MA. Stabilized helical peptides: a strategy to target protein-protein interactions. ACS Med Chem Lett. 2014 5(8):838-9. Published. Acknowledgement of federal support: yes.

- Was chosen as paper of the month by the Associate Chief of Staff of the Research Service at the Minneapolis VA Medical Center.

Poster presentation at the Annual Meeting of the Protein Society, July 22-15, 2014

POST 12-215

Development of Novel p16INK4a Peptide Mimetics as Anticancer Therapy

Marian Kratzke2, Yuk Sham3, Mark A. Klein1
[1]Medicine, Minneapolis VA Healthcare System and University of Minnesota, [2]Research Service Minneapolis VA Healthcare System, [3]Center for Drug Design, University of Minnesota, Minneapolis, Minnesota, US

Abstract

Mesothelioma is a highly fatal disease that has poorly effective therapy with dose-limiting side-effects. Low expression of the endogenous CDK4/CDK6 inhibitor, p16INK4a, has been demonstrated in up to 90% of mesothelioma tumors. Replacement of p16INK4a activity via gene therapy in laboratory models has demonstrated activity against CDK4 and CDK6, tumor response, and an increase in survival in xenograft models. Two truncated peptides (FLDTLVVLHR and DAAREGFLDTLVVLHRAGAR) derived from the 3rd anykyrin repeat of p16INK4a have been shown to exhibit similar activity to the full-length protein. An isolated 10mer peptide has also been shown to maintain its native helical structure away from its full length protein. Hypothesis: Protein-protein interactions between CDK4/6 and p16INK4a can be replicated or disrupted by engineered stabilized helical peptides identified from shortened peptides that interact with CDK4/6. Results and Discussion: We evaluated the bioactivities of several truncated peptides against CDK4/6 and mesothelioma cell lines. The IC50 values against CDK4 and CDK6 for the native peptides ranged from 14.6 μM to 21.2 μM. Peptides with a TAT-leader sequence (YGRKKRRQRRR) bound to the amino-terminal end exhibit a wider range of IC50 activities from 860 nM to 257 μM. Stabilized helical peptide derivatives (one olefin linker per peptide, i,i+4) were also studied, and the IC50 values ranged from 49 -105 μM. Against mesothelioma cell lines, the IC50 values against TAT-derived peptides ranged from 37.6 to 88.2 μM, while IC50 values for all the other peptides were > 1mM. We are currently engaged in molecular dynamics simulations to determine whether a correlation persists between the percent helicity of these peptides and its bioactivity. We anticipate such a correlation will greatly enrich our understanding in the transient structural nature of our peptides and will provide an improved platform for further design of stabilized helical peptide candidates.

Research talk at the Minneapolis VA Medical Center 1/23/14
Research talk at the University of Minnesota, 1/24/14

Websites or other Internet sites:
Nothing to report.

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on this project?

Name:	Mark Klein, M.D.
Project Role:	Principal Investigator
Research identifier:	
Nearest person month worked:	6
Contribution to project	Principal Investigator and conduct molecular dynamics studies and NMR studies
Funding Support	This grant.

Name:	Marian Kratzke, Ph.D.
Project Role:	Biological research scientist
Research identifier:	
Nearest person month worked:	9
Contribution to project	Conducted *in vitro* laboratory experiments
Funding Support	This grant.

Name:	Robert Kratzke
Project Role:	Mentor
Research identifier:	
Nearest person month worked:	1
Contribution to project	Mentor
Funding Support	None from this grant

Name:	Yuk Sham, Ph.D.
Project Role:	Collaborator
Research identifier:	

Nearest person month worked:	1
Contribution to project	Collaborator on molecular dynamics studies
Funding Support	This grant.

Has there been a change in the active other support of the PD/PI or senior/key personnel since the last reporting period?

Mark Klein, M.D., PI:
Addition of one pilot grant:

Minnesota Veterans Education and Research Foundation Klein (PI) 11/01/2014-10/31/2016
Targeting Tubulin and CK2 in Small Cell Lung Cancer
The goal of this study is to evaluate the contribution of beta-3 tubulin and CK2 to the biology of small cell lung cancer and evaluate the efficacy of targeting these two proteins alone an in combination in small cell lung cancer cells.
Effort: 1%
Amount: $20,000 over 2 years

Completion of other funding:

Transformation of Specialty Care Initiative Schorer (PI) 10/1/12-8/1/14
Veterans Affairs' Central Office
Subproject Title: Palliative Care in Head and Neck Cancer Patients
The major goal of this subproject is to determine feasibility of incorporating palliative care for head and neck cancer patients undergoing active treatment with chemotherapy and radiation.
Amount: $500,000/year, subproject $63,800/year

Yuk Sham, Ph.D., Collaborator
Addition of grant support beside the current grant.

Agency: U of MN, Center for Drug Design
Type: Seed Grant
Title: Second generation non-beta-lactam beta-lactamase inhibitors as a topical treatment for infected wound and drug resistant Neisseria gonorrhoeae
PI: Yuk Sham
Co-PI: Steve Patterson
Consultant: Swati More
Total: $177K
Duration: 07/15/14 - 09/14/16

Funding Agency: Nanyang Technological University Tier 1 Grant Program, Singapore
Type: Career Development Grant (Peer-Reviewed)
Title: Photochromic Aptamer Switch Assay: A Universal Bioassay Device
PI: Terry Steele
Co-PI: Yuk Sham, Robert Marks, Vladislav Papper
Total: $200K
Duration: 01/01/14 - 12/31/15

What other organizations were involved as partners?

University of Minnesota
Minneapolis, Minnesota

Facilities: use of the Supercomputing Institute and NMR facility
Collaboration: Dr. Yuk Sham, Center for Drug Design

SPECIAL REPORTING REQUIREMENTS

Nothing to report.

APPENDICES

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Stabilized Helical Peptides: A Strategy to Target Protein−Protein Interactions

Mark A. Klein*

Minneapolis VA Health Care System, 1 Veterans' Drive, Minneapolis, Minnesota 55417, United States, and Division of Hematology, Oncology, and Transplantation, Department of Medicine, University of Minnesota, Minneapolis, Minnesota 55455, United States

ABSTRACT: Protein−protein interactions are critical for cell proliferation, differentiation, and function. Peptides hold great promise for clinical applications focused on targeting protein−protein interactions. Advantages of peptides include a large chemical space and potential diversity of sequences and structures. However, peptides do present well-known challenges for drug development. Progress has been made in the development of stabilizing alpha helices for potential therapeutic applications. Advantages and disadvantages of different methods of helical peptide stabilization are discussed.

KEYWORDS: *stabilized peptide, α helix, protein−protein interactions*

In the last two decades, there has been great progress in new therapies for several disease types. The highest number of new drugs have been for oncology. In oncology, there has been a new era in treatment. Many would consider the introduction of imatinib mesylate (Gleevec) for chronic myelogenous leukemia as the start of this new era.[1] That discovery led to numerous efforts in developing new small molecule kinase inhibitors with the goal of finding the next "Gleevec". Many of these small molecule inhibitors follow Lipinski's "Rule of 5s".[2] While there have been successes in the development of small molecule inhibition of enzymes important in cell function and growth, many small molecule inhibitors have not been as effective as desired when given as single agent therapy. On the basis of these results, other strategies are needed to achieve better disease control across the spectrum.

■ TARGETING PROTEIN−PROTEIN INTERACTIONS

Strategies to target protein−protein interactions are numerous. One has been to enlist combination therapy utilizing small molecule inhibitors in combination. The vast majority of small molecule inhibitors are enzyme inhibitors. One of the disadvantages in targeting enzyme sites is that the sites may be conserved among different enzymes of the same family. Much of the specificity in cellular pathways includes the myriad protein−protein interactions that occur in the cell. Interest in protein−protein interactions in the cell recently has led to coining of the term "interactome".[3] One estimate is that there may be about 650,000 significant protein−protein interactions in the cell.[3] When thinking about the complexity of intracellular protein interactions in addition to the dynamics within each protein that may affect those protein−protein interactions, the number of potential targets seems to increase exponentially. However, targeting protein−protein interactions has been thought by some to be "undruggable". In light of recent successes and the vast potential for the development of more effective, less toxic therapies, a significant research and development effort seems to be in order.

Previous efforts have included different strategies. The nature of protein−protein interactions have been thought to be difficult to target due to the nature of the binding sites. Namely, the sites tend to be hydrophobic and shallow.[4] However, the discovery of "hot spots" has led to the hypothesis that disrupting protein−protein interactions does not need to target the entire surface but rather instead only a few, key smaller sites. Preclinical work in Bcl-2 inhibition led to development of ABT-263 and related compounds.[5] The thrombopoietin agonist eltrombopag (utilized in immune thrombocytopenic purpura) is an example of a small molecule peptidomimetic that mimics the activity of a larger protein. Another intriguing approach has been efforts examining modification of peptides to target protein−protein interactions.

■ STABILIZING HELICES

Small peptides (often consisting of a domain or smaller in size) have been evaluated extensively. The drawbacks of peptides as drugs are well-known. Native peptides outside of a parent structure can be quite sensitive to protease degradation. In addition, oral absorption is quite difficult to achieve. One of the most utilized peptides in the clinic is octretotide, and it has been used in several conditions, including carcinoid syndrome.[6] This peptide is able to be delivered subcutaneously. The cyclic nature of this peptide makes it more "drug-like" than a native, nonmodified peptide would be. Research into other modifications of peptides to get past the drawbacks listed above has increased over the last 10 years. While there are numerous studies that have been done, three approaches will be discussed here.

Probably the best known is the use of "stapled peptides" originally described by Walensky and Verdine.[7] In 2000, Verdine and colleagues first reported the synthesis of stabilized peptides,[8] and in 2004, Walensky, Verdine, and Korsmeyer published their results on the effects of stabilizing pro-apoptotic BH3-mimetic peptides.[7] Taking an isolated peptide sequence out of its larger parent protein is thought to significantly decrease the propensity to fold into the same secondary structure that is found in the parent protein. However, such

Published: June 23, 2014

peptides usually are in an array of conformations; it is a matter of the population of structures in a conformation that is more biologically relevant. Using olefin tethers, peptides were stabilized via links at positions i,i + 4 or i,i + 7. The stabilized peptides had increased propensity toward helicity, were able to be taken up by cells, and were effective *in vivo* against leukemia cells. Other groups have started to utilize this approach. This approach is quite promising, but it has engendered some debate. There have been few pharmacokinetic studies published, so how broadly this approach will be applicable in broader drug discovery is unclear. Positioning of the staples and the stereochemistry of the nonnatural amino acids introduced into the sequence to act as the olefin base is being worked out. The obvious concern is that the olefin linker could interfere with the key residues important for target binding; this could negate the benefits of tethering. The Genentech group was unable to reproduce some of Walensky et al.'s results. As is common in scientific endeavors, it is unclear why there are discrepant results obtained by different laboratories. (Identification of the reasons for the discrepancy could be very useful to the scientific community. Walensky et al. have proposed reasonable possibilities.)

As there are multiple ways to potentially stabilize helical peptides, other approaches have been studied. Gellman's group has extensively studied the introduction of beta peptides into peptide backbones (containing an extra carbon in the backbone) in order to determine the effects of backbone modification.[9] An advantage to this approach is that the side chains are not blocked or removed by a tethering functional group. It also may remove a separate chemical modification step, which may not be trivial in thinking downstream about manufacturing. Several alpha/beta sequences have been studied ($\alpha\beta\alpha\beta$, $\alpha\alpha\beta\alpha\alpha\alpha\beta$, $\alpha\alpha\beta\alpha\alpha\beta$, $\alpha\alpha\alpha\beta\alpha\alpha\alpha\beta$, etc.). This approach has been utilized to identify analogues of the Bim BH3 domain that are able to bind Bcl-2 family proteins and induce apoptosis in mice embryonic fibroblast extracts.

A third approach has been to use a hydrogen bond surrogate approach.[10] This includes mimicking the hydrogen bonds that stabilize the helix backbone. An advantage to this approach includes not requiring side chain substitution. A potential disadvantage is that thus far, only the amino terminal is likely amenable to this technique. While that may be enough to overcome the entropic penalty to fold a peptide into a helix, one can envision that the C-terminus is potentially left vulnerable in comparison to the other techniques discussed above.

MOVING FORWARD

A daunting advantage of the utilization of peptides as drugs includes the immense number of possible different peptides that can be developed. As there are 20 amino acids that may be substituted in each position, a 10 amino acid has 1.024×10^{13} possible sequences. A 30 amino acid has about 1×10^{37} possible sequences. It is evident that with current technology screening every possible combination of peptides, or even a significant fraction of that, is not possible. Other strategies are needed. As computational power increases, it may be possible to better model peptides. Much of the work described above has occurred in the past 10 years or so. It is exciting to see what the next 10 years hold in this field.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mark.klein2@va.gov.

Funding

The author has funding from the Department of Defense.

Notes

Views expressed in this editorial are those of the author and not necessarily the views of the ACS.

The authors declare no competing financial interest.

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